

Alterations of mucosal microbiota in the colon of patients with inflammatory bowel disease revealed by real time polymerase chain reaction amplification of 16S ribosomal ribonucleic acid

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Background & objectives: Alterations in microbial communities closely associated with the intestinal mucosa are likely to be important in the pathogenesis of inflammatory bowel disease (IBD). We examined the abundance of specific microbial populations in colonic mucosa of patients with ulcerative colitis (UC), Crohn's disease (CD) and controls using reverse transcription quantitative polymerase chain reaction (RT-qPCR) amplification of 16S ribosomal ribonucleic acid (16S rRNA).

Methods: RNA was extracted from colonic mucosal biopsies of patients with UC (32), CD (28) and patients undergoing screening colonoscopy (controls), and subjected to RT-qPCR using primers targeted at 16S rRNA sequences specific to selected microbial populations.

Results: *Bacteroides-Prevotella-Porphyromonas* group and *Enterobacteriaceae* were the most abundant mucosal microbiota. *Bacteroides* and *Lactobacillus* abundance was greater in UC patients compared with controls or CD. *Escherichia coli* abundance was increased in UC compared with controls. *Clostridium coccoides* group and *C. leptum* group abundances were reduced in CD compared with controls. Microbial population did not differ between diseased and adjacent normal mucosa, or between untreated patients and those already on medical treatment. The Firmicutes to Bacteroidetes ratio was significantly decreased in both UC and CD compared with controls, indicative of a dysbiosis in both conditions.

Interpretation & conclusions: Dysbiosis appears to be a primary feature in both CD and UC. Microbiome-directed interventions are likely to be appropriate in therapy of IBD.

Key words Bacteroidetes - Crohn's disease - dysbiosis - Firmicutes - microbiota - ulcerative colitis

The inflammatory bowel disease (IBD), ulcerative colitis (UC) and Crohn's disease (CD) is thought to represent abnormal innate inflammatory reactions occurring in response to normal constituents of the gut microbiota in genetically predisposed individuals. The gut contains trillions of microbes which affect human

health in many ways through effects on nutrition, metabolism and immunity¹. The gut microbiota influence innate and adaptive immune responses in the intestine. Some of the microbial populations dampen immune and inflammatory responses through amplification of regulatory immune cells,

while others induce inflammatory reactions². IBD has been associated with a breakdown in the balance between putative species of protective versus harmful intestinal bacteria, a phenomenon that has been termed dysbiosis³.

Most of the studies on dysbiosis in IBD have examined the balance of specific microbial populations in the faeces. While the bulk of the gastrointestinal microbiota reside within the lumen, the subset that resides in the outer mucus layer covering the epithelial surface is likely to be most important in the gut microbial interaction with the innate immune system⁴, and therefore, it is most likely to have a bearing on the pathogenesis of IBD. Several studies, including one from India, have examined colonic mucosal bacterial populations in IBD and demonstrated alterations in the balance between putative beneficial and harmful bacterial populations^{5,6}. The mucosa-associated bacteria are difficult to study because of their low numbers. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) targeting 16S ribosomal RNA (rRNA) molecules is more than 100-fold sensitive and specific than qPCR alone in quantitating the microbial population because of the high copy number of targeted rRNA molecules⁷. Moreover, measuring 16s rRNA rather than the DNA (16S rRNA gene) identifies actively transcribing bacteria, which are more likely to affect intestinal immune reactions and thus have a greater bearing on human health than inactive or dead bacteria⁸.

The primary objective of the present study was to determine whether there were alterations in specific actively transcribing bacterial populations in the colonic mucosa of IBD patients compared to control volunteers. The secondary objectives were to determine whether there were differences in the actively transcribing bacterial populations between normal and abnormal mucosa of IBD patients, and to determine whether the balance and distribution of the microbial populations were influenced by the severity of mucosal inflammation or by medical therapy.

Material & Methods

The participants were recruited from the Gastroenterology and Inflammatory Bowel Disease clinics of the Christian Medical College, Vellore, Tamil Nadu, India, from December 2008 to December 2010. Consecutive patients with IBD undergoing colonoscopy were recruited as the case group, while controls were recruited from patients with haemorrhoidal bleeding

referred for colonoscopy to exclude neoplasia. IBD diagnosis and characterization was based on standard consensus criteria⁹. Patients were investigated as per the usual clinical protocol of the department.

The participants provided written informed consent for the additional biopsies that were taken for study. The study protocol was approved by the Institutional Review Board of the Christian Medical College, Vellore.

Colonoscopies were performed after colonic lavage using orally administered polyethylene glycol-electrolyte solution. Mucosal biopsy samples were obtained using standard biopsy forceps from endoscopically normal mucosa of the colon in controls and from abnormal or ulcerated mucosa in patients with IBD. In patients with CD and in UC patients with limited disease, apparently normal mucosa at least 5 cm from endoscopically abnormal areas was also biopsied for the study. Mucosal biopsies were immediately washed in sterile saline, snap-frozen in liquid nitrogen, and stored at -80°C until processing. Zoetendal *et al*¹⁰ examined mucosal biopsies from different regions of the colon and showed that the mucosal bacterial community in the human colon was uniformly distributed throughout the colon with little variation by location. In our study, since biopsies from IBD patients included both normal and ulcerated mucosa from different regions of the colon, the normal and ulcerated biopsies were matched by region of the colon.

Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR): RNA was extracted from the biopsies using Tri reagent (Sigma, USA), following manufacturer's instructions. RNA concentration in the samples were measured by spectrometry (Nanodrop, Thermo Scientific, USA). RNA samples (250 ng per biopsy sample) were converted to cDNA, using reverse transcriptase core kit (Eurogentec, Liege, Belgium). RNase pretreatment was not used⁷. The reverse transcription reaction mix was prepared on ice and contained final concentration of 1X buffer, 5mM MgCl₂, 500 μM of each deoxy nucleotide phosphate (dNTPs), 2.5 μM random nonamer, 0.4 U/μl of RNase inhibitor, 1.25 U/μl of EuroScript RT, 250 ng of total RNA and RNase free water to adjust the final volume to 10 μl. The cDNA conversion was carried out in a PTC-150 MiniCycler (MJ Research, USA) using a cycling protocol with an initial step for 10 min at 25 °C, followed by a reverse transcriptase step for 30 min at 48 °C and a final inactivation step for 5 min at 95 °C.

Diluted cDNA samples were then amplified in quantitative PCR. Amplification and detection were carried out in 96-well plates with Mesa Green qPCR MasterMix Plus for SYBR® Assay I dTTP (Catalog number RT-SY2X-03+NR WOUN, Eurogentec, Liege, Belgium) to quantify total bacteria and the specific bacterial populations listed in Table I. Each reaction was done in duplicate in a final volume of 20 µl with 2X MasterMix, 250 nM final concentration of each primer, and 2 µl of DNA sample. Quantification was performed in a real time PCR instrument, the Chromo 4 thermocycler coupled to a fluorescence detector (Biorad, USA) under the following conditions: initial denaturation at 95°C for 5 min followed by 45 cycles of 95°C for 20 sec, annealing temperature as shown in Table II for each bacterial population, and final elongation at 72°C for 30 sec. Fluorescence quantitation was analyzed using the Opticon monitor system software v. 3 (Biorad, USA). A dissociation step was added and melting curves were analyzed to confirm the identity and fidelity of amplification of SYBR Green products.

Details of the 16S rRNA-targeted primers used in this study and their specificities are described in Table I¹¹⁻¹⁸. These primers were purchased from Bioserve Biotechnologies (Hyderabad, India). The Opticon 3.1 (BioRad, Hercules, CA, USA) software, provided with the instrument, plotted the rate of change of the relative fluorescence units (RFU) with reference to time (T) (-d (RFU)/dT) on the Y-axis versus the temperature on the X-axis, with the curve peaking at the melting temperature. Quantification was based on the fluorescence intensity obtained from the intercalated SYBR Green dye. The cycle number at which the signal was first detected - the threshold cycle (Ct) - correlated with the original concentration of DNA template. DNA copy was not expressed as absolute number but was expressed as the relative abundance of the specified microbial population, being calculated from the Ct at which DNA for each target bacterial population was detected relative to the Ct at which universal bacterial DNA was detected during amplification, using the formula $2^{-\Delta Ct}$, where $\Delta Ct = Ct [\text{Bacteria of interest}] - Ct [\text{Universal bacteria}]$, and expressed by the software as the relative fold difference. This relative quantification was done automatically by the Opticon 3.1 software. The relative abundance of the target bacterial population was thus the ratio of the target bacterial rRNA compared to universal rRNA, providing a quantitative comparison between different

samples. The ratio calculated for each of the duplicate samples always correlated well with the other, with an adjusted R² in the region of 0.80¹¹.

Statistical analysis: The quantitative PCR was done with two technical replicates for each sample. The mean of the relative difference was taken for duplicate. The relative difference for each bacterial population in each study group (UC, CD, control) was expressed as mean±SEM, and the significance of differences between groups was assessed using unpaired t test with Welch correction for unequal variances.

Results

Thirty control participants, 28 patients with CD and 32 patients with UC were included in the study. Their demographic and clinical characteristics are described in Table II.

Differences in mucosal microbiota between controls and IBD patients: The two major microbial communities associated with the mucosa were the *Bacteroides-Prevotella-Porphyromonas* group and the family *Enterobacteriaceae*. The *Bacteroides-Prevotella-Porphyromonas* group was significantly more abundant in UC compared with controls as well as CD (Fig. 1). *Enterobacteriaceae*, a major aerobic bacterial group, was not significantly different between the groups. However *Escherichia coli*, a constituent of *Enterobacteriaceae*, was significantly more abundant in the mucosa of patients with UC. *Lactobacillus* group bacteria were also significantly increased in UC compared with controls or CD (Fig. 1). *Clostridium coccoides-Eubacterium rectale* group was significantly less abundant in CD mucosa compared with controls and *Clostridium leptum* group was significantly less abundant in CD compared to UC mucosa (Fig. 1). The other microbial populations tested were not significantly different between the study groups.

Differences in mucosal microbiota between normal and inflamed areas of the colon: Mucosal inflammation or ulceration may potentially alter the abundance of microbial population by altering the microenvironment necessary for the association of these microbiota with the mucosa. The relative abundance of mucosal microbiota between normal and inflamed colonic mucosa was compared in patients with IBD (Fig. 2). In 24 patients with CD, paired biopsies were available from both normal and inflamed areas of the colon. There were no significant differences in microbiota composition between normal and inflamed areas (Table III). Paired

Table I. Primers used in this study and amplicon sizes

Primer/specificity	Forward sequence (5'-3')	Reverse sequence (5'-3')	Annealing temperature (°C)	Size (bp)	Ref.
Universal/domain bacteria	TCCTACGGGAGGCAGCAGT	GGACTACCAGGGTATCTAATCCTGTT	61	466	11
<i>Bifidobacterium</i> /genus	CGGGTGAGTAATGCGTGACC	TGATAGGACGCGACCCCA	61	122	12
<i>Lactobacillus</i> /group	AGCAGTAGGGAATCTTCCA	CGCCACTGGTGTTCYTCCAIATA	60	353	12
<i>Bacteriodes-Prevotella</i> /group	GGTGTCCGGCTTAAGTGCCAT	CGGACGTAAGGGCCGTGC	60	140	11
<i>Enterobacteriaceae-1</i> /genus	CATTGACGTTACCCGCAGAAG	CTCTACGAGACTCAAGCTTGC	56	196	13
<i>Clostridium coccooides-Eubacterium rectale</i> /group	CGGTACCTGACTAAGAAGC	AGTTTCATTCTTGCGAAC	59	412	14
<i>Clostridium leptum</i> /group	GCACAAAGCAGTGGAGT	CTTCCTCCGTTTTGTCAA	58	239	14
<i>Desulfovibrio</i> /genus	CCGTAGATACTGGAGGAACATCAG	ACAICTAGCAATCCATCGTTTACAGC	60	136	15
<i>Enterobacteriaceae-2</i> /genus	CAIGCCCGGTGATGAAGAA	CGGGTAACGTCAATGAGCAAA	59	96	17
<i>Faecalibacterium prausnitzii</i> /species	GGAGGATTGACCCCTTCAGT	CTGGTCCCGAAGAAAACACAT	61	203	18

Table II. Demographic and clinical characteristics of participants

	Control	Crohn's disease	Ulcerative colitis
N	30	28	32
Age (yr) mean \pm SEM	39.6 \pm 2.6	32.4 \pm 2	37.9 \pm 2.5
Male, n (%)	22 (77)	20 (71)	16 (50)
Extent		I 3 IC 11 C 13 U 1	PR 7 LS 11 PC 14
Treatment			
Steroid	0	7	14
Purine analogue	0	8	3
Methotrexate	0	0	1
Mesalazine	0	14	19
Sulphasalazine	0	0	7
Antibiotics	0	0	0

I, ileal; IC, ileocolonic; C, colonic; PR, proctitis; LS, left-sided colitis; PC, pancolitis; U, upper GI tract

biopsies from normal and inflamed areas were available in 18 patients with UC. Again, there were no significant difference in microbiota composition between normal and inflamed mucosa (Table III).

Effect of prior treatment on mucosal bacteria in IBD: Medical therapy of IBD can potentially alter microbiota composition. We compared mucosal bacteria in treatment-naïve IBD (CD and UC) patients with those who were receiving treatment at the time of biopsy. There were no significant differences in the mucosal microbiota between the two groups, except

for *Clostridium coccoides-Eubacterium rectale* that was significantly more abundant in patients on therapy (Fig. 3).

Ratio of Firmicutes to Bacteroidetes in the mucosa: *Clostridium coccoides-Eubacterium rectale* (cluster XIV) and *Clostridium leptum* (cluster IV) groups represent the major constituents of phylum Firmicutes among the colonic microbiota, while *Bacteroides-Prevotella-Porphyromonas* group represents the major constituent of phylum Bacteroidetes in the colonic microbiota. The ratio of Firmicutes to Bacteroidetes has been used to express the degree of dysbiosis in IBD. We, therefore, examined the ratio of *Clostridium* (sum of the two *Clostridium* clusters measured) to *Bacteroides* among the mucosal microbiota as a measure of dysbiosis. This ratio was significantly reduced in both forms of IBD, in both inflamed and normal mucosa, and regardless of treatment (Fig. 4).

Discussion

We report that *Bacteroides-Prevotella-Porphyromonas* and *Enterobacteriaceae* were the most abundant microbial population associated with the colonic mucosa. By contrast, Firmicutes (principally clostridia) which are the most abundant constituents of the colonic luminal microbiota, were much less abundant in the mucosa. Importantly, our findings confirmed the existence of a dysbiosis in the mucosa in IBD patients that did not appear to be secondary to the presence of mucosal inflammation.

Clostridium coccoides group, including *Eubacterium rectale*, constitutes *Clostridium* cluster XIV, while *Clostridium leptum* group, including *Faecalibacterium prausnitzii*, is the major constituent of *Clostridium* cluster IV. Changes described in the

Table III. Bacterial populations in biopsies from normal areas and from inflamed areas in patients with Crohn's disease and ulcerative colitis

Bacterial community/group	Crohn's disease (n=24)		Ulcerative colitis (n=18)	
	Normal	Inflamed	Normal	Inflamed
<i>Bifidobacterium</i>	0.005 \pm 0.002	0.004 \pm 0.001	0.007 \pm 0.002	0.01 \pm 0.003
<i>Lactobacillus</i>	0.007 \pm 0.004	0.002 \pm 0.0005	0.01 \pm .005	0.11 \pm 0.07
<i>Bacteroides-Prevotella</i> group	0.35 \pm 0.05	0.29 \pm 0.05	0.45 \pm 0.07	0.44 \pm 0.04
<i>Enterobacteriaceae</i>	0.48 \pm 0.08	0.49 \pm 0.07	0.64 \pm 0.15	0.93 \pm 0.21
<i>Clostridium Coccoides-Eubacterium Rectale</i> group	0.03 \pm 0.007	0.02 \pm 0.005	0.04 \pm 0.01	0.03 \pm 0.007
<i>Clostridium leptum</i>	0.005 \pm 0.001	0.002 \pm 0.0007	0.01 \pm 0.0005	0.11 \pm 0.07

The values are mean \pm SEM of the relative abundance of each bacterial population compared to overall total bacteria

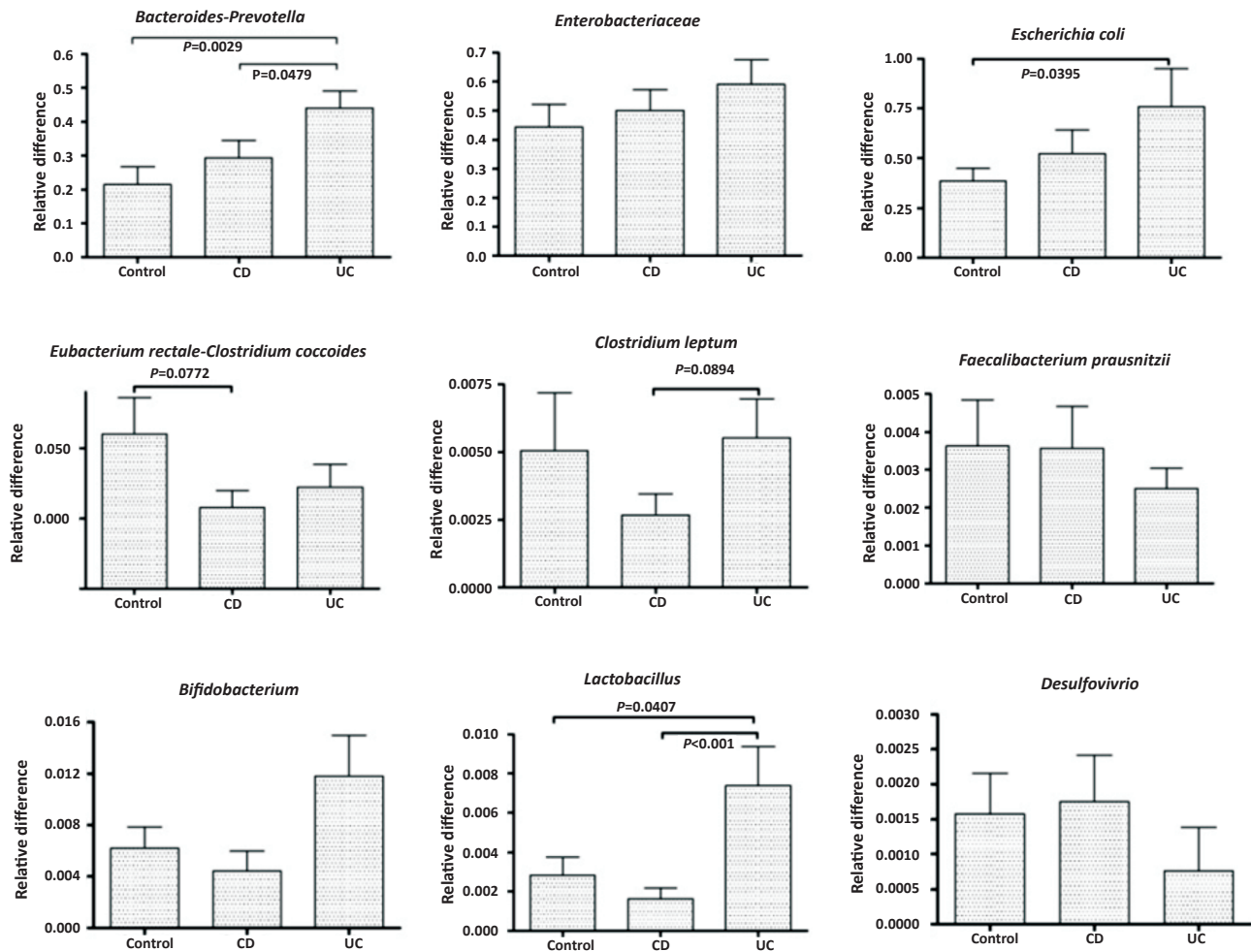


Fig. 1. Quantitative representation of the various mucosa-associated bacterial populations in controls, patients with Crohn's disease (CD) and patients with ulcerative colitis (UC). The bars represent mean \pm SEM of the relative difference which is the fold amplification of sequences for the target population relative to amplification of universal bacterial domain sequences. Significant differences (ANOVA with post-hoc Dunnett's test) are shown.

faeces in CD include reductions in number of bacteria belonging to these two clusters, which are important in carbohydrate fermentation and short chain fatty acid (particularly butyrate) production¹⁹. Changes described in the faecal microbiota, at the phylum level, include increased abundance of Bacteroidetes and Proteobacteria, and reduced abundance of Firmicutes in the faeces of patients with CD²⁰.

In contrast to the above studies of faecal microbiota, studies of the mucosal microbiota in IBD have used a variety of approaches and resulted in variable findings. Invasive-adherent *E. coli* have been reported in the ileal mucosal biopsies of patients with ileal CD²¹. Hartley

*et al*²² isolated greater numbers of *Bacteroides* species from rectal biopsies of patients with active UC than from patients with inactive disease. Using molecular techniques, Bibiloni *et al*²³ reported an increase in unclassified bacteria belonging to phylum Bacteroidetes in biopsies from CD patients and an increase in *Porphyromonas* species in biopsies from UC patients. Verma *et al*⁵ showed that Bacteroidetes population decreased in IBD patients compared to controls. In the present study, *Bacteroides-Prevotella-Porphyromonas* group was increased in the mucosa of UC patients compared with CD and controls. The differences between the two studies, both based in India, are likely

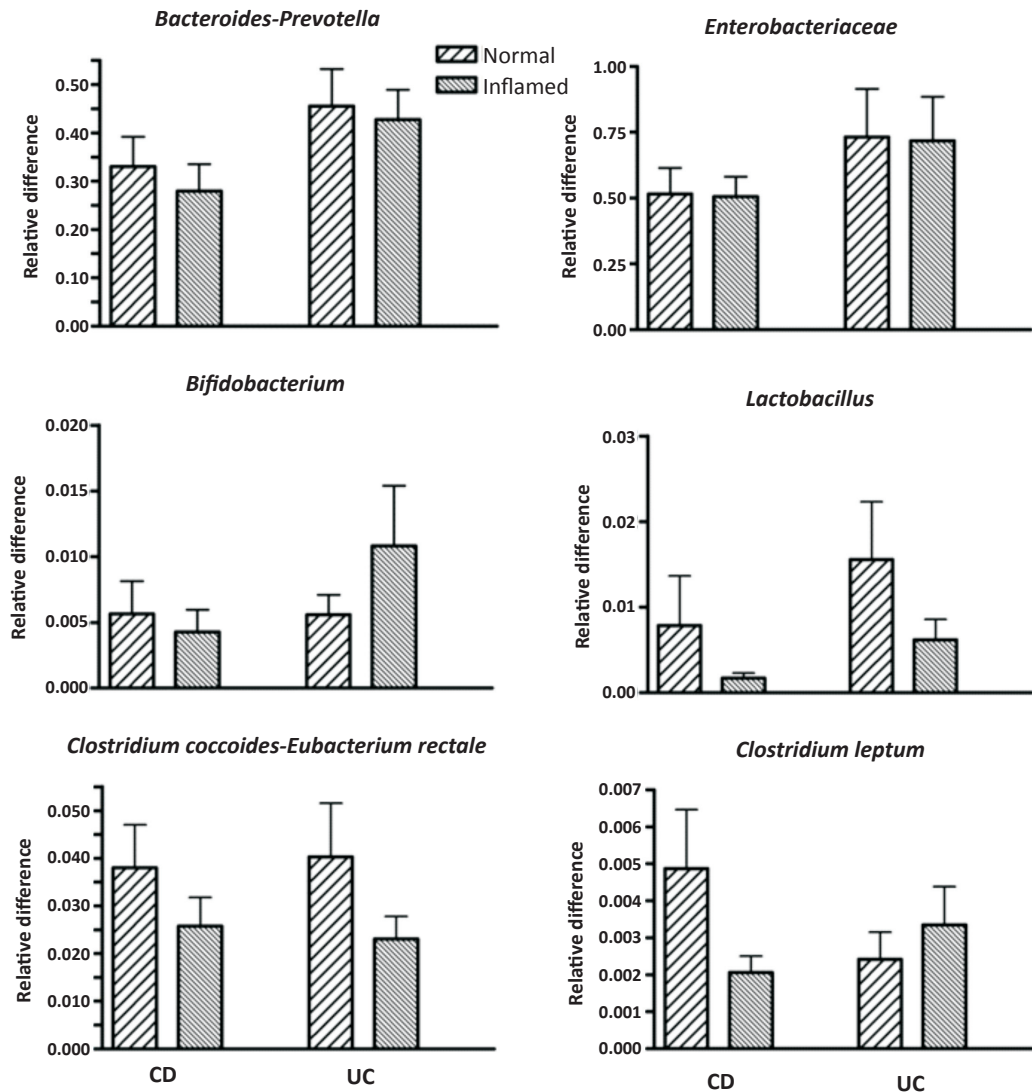


Fig. 2. Bacterial populations in ulcerated and normal colonic mucosa of patients with Crohn's disease (CD) and ulcerative colitis (UC). Bars represent mean \pm SEM of the relative difference (fold amplification with reference to amplification of universal bacterial domain sequences). None of the differences between normal and ulcerated mucosa for each bacterial population was significant.

to reflect the use of different primers to quantitate the bacterial population in these two studies. As in the above Indian study⁵, we also found decrease in the numbers of *Clostridium* in the mucosa of patients with CD. Swidsinki *et al*⁴ investigated the mucosal bacteria by *in situ* hybridization in biopsies where the mucus layer was retained, and found reduced abundance of bacteria belonging to the *C. coccoides-E. rectale* group and increased abundance of *Bacteroides* in patients with IBD compared to those with irritable bowel syndrome. Reduced abundance of *Faecalibacterium prausnitzii*

in ileocolonic mucosal biopsies from patients with CD has also been reported²⁵. Increased populations of phylum Bacteroidetes and reduced populations of phylum Firmicutes in the mucosa of IBD, especially CD, patients have also been reported. Quantitation of 16S rRNA in mucosal biopsies suggested that *E. coli* were both abundant and transcriptionally very active in both forms of IBD, while transcription of Actinobacteria and Firmicutes was suppressed in CD²⁶. The present study was generally consistent with the above studies, showing an increase in the relative

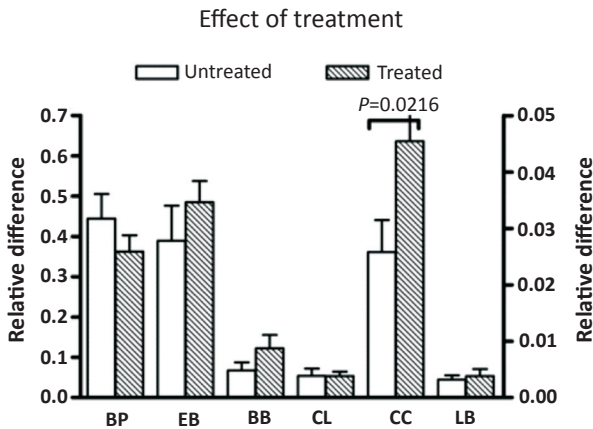


Fig. 3. Bacterial populations in ulcerated mucosa of treatment-naïve or treated patients with IBD. Bars represent mean \pm SEM of the relative difference which is the fold amplification of sequences for the target population relative to amplification of universal bacterial domain sequences. Significant differences (unpaired t test with Welch's correction) are indicated in the Figure. *Bacteroides-Prevotella-Porphyromonas* and *Enterobacteriaceae* are depicted on the left Y-axis; the remaining bacterial populations are depicted on the right Y-axis. BP, *Bacteroides-Prevotella-Porphyromonas*; EB, *Enterobacteriaceae*; BB, *Bifidobacteria*; CL, *Clostridium leptum*; CC, *Clostridium coccoides*; LB, *Lactobacillus*.

abundance of *Bacteroides* and *E. coli* in the mucosa of UC patients and decrease in relative abundance of *Clostridium* clusters XIV and IV in CD. Differences between studies may relate to differences in techniques for identification and quantitation, differences in diet of the individuals, and to restricted sample sizes in some studies.

The present study did not find significant differences in the mucosa-associated bacteria between apparently normal and inflamed mucosa in patients with IBD. In an earlier study comparing ulcerated and non-ulcerated mucosa in CD and UC, increases in Bacteroidetes and reduction in Firmicutes were more likely to be observed in ulcerated mucosa than in non-ulcerated mucosa; however, the numbers studied were small and there was considerable inter-individual variability²⁷. In another study, bacterial community diversity was found to be less in ulcerated mucosa compared to non-ulcerated mucosa; however, that study did not attempt to generate microbial quantitative data²⁸. The present study used RT-qPCR to quantitate specific microbial populations, and did not show significant differences in relative microbial abundance between ulcerated and non-ulcerated areas.

Bacteroides are important commensal bacteria that have been implicated in the pathogenesis of inflammatory bowel disease²⁹. On the other hand, microbes belonging to the *Clostridium* clusters XIV and IV are important and major constituents of the normal colonic microbiota. The alteration in the ratio between *Clostridium* species which are considered beneficial and *Bacteroides* which are considered aggressive, is an indicator of dysbiosis in patients with IBD. The Firmicutes to Bacteroidetes ratio in faeces is very similar to the above and has been considered as an indicator of human gut microbiota status. An increase in Firmicutes characterizes obesity¹ while an increase in Bacteroidetes has been reported to occur with ageing³⁰. While one study suggests that the ratio of *Faecalibacterium prausnitzii* to *E. coli* is the important factor in dysbiosis, it is possible that the specific microbial imbalance may vary in different communities and countries. Understanding this better will obviously be of importance when considering therapies to counter dysbiosis. Another study done from north India which looked at mucosal microbiota in ulcerated areas, did not examine bacterial populations in normal mucosa of IBD patients⁵.

In conclusion, there were alterations in the mucosa-associated microbiota in both UC and CD characterized by reduction in abundance of microbes belonging to phylum Firmicutes relative to microbes belonging to phylum Bacteroidetes. The microbial populations did

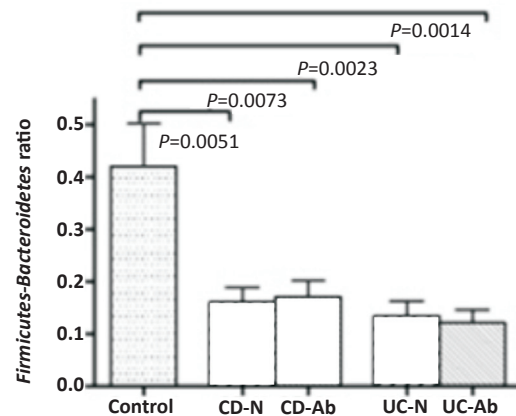


Fig. 4. Ratio of Firmicutes (*C. coccoides* plus *C. leptum*) to Bacteroidetes in the mucosal microbiota. The Figure shows the ratio in patients with CD and UC. CD-N and CD-Ab refer to Crohn's disease normal and abnormal mucosa. UC-N and UC-Ab refer to ulcerative colitis normal and abnormal mucosa. Significant *P* values are shown above the bars in the Figure. None of the other differences was significant. IBD biopsies showed a significantly lower ratio compared with controls.

not significantly differ between normal and inflamed mucosa or between untreated and treated patients. These findings suggested that dysbiosis was a primary feature of both forms of IBD and that microbiome-directed interventions could be appropriate targets for therapy of IBD.

Conflicts of interest: None.

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